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An epigenome-wide association study meta-analysis of educational attainment

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IMMEDIATE COMMUNICATION

An epigenome-wide association study meta-analysis of educational attainment

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The epigenome is associated with biological factors, such as disease status, and environmental factors, such as smoking, alcohol consumption and body mass index. Although there is a widespread perception that environmental influences on the epigenome are pervasive and profound, there has been little evidence to date in humans with respect to environmental factors that are biologically distal. Here we provide evidence on the associations between epigenetic modifications—in our case, CpG methylation—and educational attainment (EA), a biologically distal environmental factor that is arguably among the most important life-shaping experiences for individuals. Specifically, we report the results of an epigenome-wide association study meta-analysis of EA based on data from 27 cohort studies with a total of 10 767 individuals. We find nine CpG probes significantly associated with EA. However, robustness analyses show that all nine probes have previously been found to be associated with smoking. Only two

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associations remain when we perform a sensitivity analysis in the subset of never-smokers, and these two probes are known to be strongly associated with maternal smoking during pregnancy, and thus their association with EA could be due to correlation between EA and maternal smoking. Moreover, the effect sizes of the associations with EA are far smaller than the known associations with the biologically proximal environmental factors alcohol consumption, body mass index, smoking and maternal smoking during pregnancy. Follow-up analyses that combine the effects of many probes also point to small methylation associations with EA that are highly correlated with the combined effects of smoking. If our findings regarding EA can be generalized to other biologically distal environmental factors, then they cast doubt on the hypothesis that such factors have large effects on the epigenome.

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INTRODUCTION

The epigenome has been shown to be associated with biological factors such as disease status.^{1,2} Although there is a widespread perception in the social sciences that a variety of social environmental factors have an effect on the epigenome,^{3–10} virtually all of the replicated evidence to date in humans relates to environmental factors that have a fairly direct biological impact, such as smoking,^{11–13} alcohol consumption^{14,15} and excess energy intake resulting in increased body mass index (BMI).^{16,17} Here we study the associations between epigenetic modifications—specifically, the methylation of cytosine–guanine pairs connected by a phosphate link (CpG methylation)—and educational attainment (EA). EA is biologically distal, and yet it is arguably among the most important life-shaping experiences for individuals in modern societies. EA therefore provides a useful test case for whether and to what extent biologically distal environmental factors may affect the epigenome.

In this paper, we report the results of a large-scale epigenome-wide association study (EWAS) meta-analysis of EA. By meta-analyzing harmonized EWAS results across 27 cohort studies, we were able to attain an overall sample size of 10 767 individuals of recent European ancestry, making this study one of the largest EWAS to date.^{13,15,18} A large sample size is important because little is known about plausible EWAS effect sizes for complex phenotypes such as EA, and an underpowered analysis would run a high risk of both false negatives and false positives.^{19,20}

As is standard in EWAS, we used data on CpG DNA methylation. This is the most widely studied epigenetic mark in large cohort studies.¹ Methylation level was measured by the beta value, which is the proportion of methylated molecules at each CpG locus, a continuous variable ranging between 0 and 1.²¹ The Illumina 450k Bead Chip measures methylation levels at >480 000 loci in human DNA and has been used in many cohort studies.¹

We report results from two common methods for the analysis of such methylation data sets. The first main analysis is an EWAS, which considers regression models for each CpG locus with EA. Using the EWAS results, we then performed a series of follow-up analyses: enrichment analyses, prediction analyses, correlation with tissue-specific methylation, and gene-expression analysis (Supplementary Note). The second main analysis uses the ‘epigenetic clock’^{22,23} method, which employs a weighted linear combination of a subset of probes (that is, measured CpG methylation loci) to predict an individual’s so-called ‘biological age’. The resulting variable can then be linked to phenotypes and health outcomes.

EWAS studies to date have found associations between DNA methylation and, for example, smoking,^{11,12} BMI,^{16,24} traumatic stress,²⁵ alcohol consumption^{14,26} and cancer.^{2,27} In prior work, an age-accelerated epigenetic clock (that is, an older biological than chronological age) has been linked to increased mortality risk,²⁸ poorer cognitive and physical health,²⁹ greater Alzheimer’s

disease pathology,³⁰ Down’s syndrome,³¹ high lifetime stress³² and lower income.³³

METHODS

Participating cohorts

We obtained summary-level association statistics from 27 independent cohort studies across 15 cohorts located in Europe, the United States and Australia (Supplementary Table S1.1). The total sample size comprised 10 767 individuals of recent European ancestry. All participants provided written informed consent, and all contributing cohorts confirmed compliance with their Local Research Ethics Committees or Institutional Review Boards.

EA measures

Following earlier work of the Social Science Genetic Association Consortium,^{34,35} EA was harmonized across cohorts. The EA variable is defined in accordance with the ISCED 1997 classification (UNESCO), leading to seven categories of EA that are internationally comparable. The categories are translated into US years-of-schooling equivalents, which have a quantitative interpretation (Supplementary Tables S1.2 and S1.3).

Participant inclusion criteria

To be included in the current analysis, participants had to satisfy six criteria: (1) participants were assessed for EA at or after 25 years of age; (2) participants were of European ancestry; (3) all relevant covariate data were available for each participant; (4) participants passed the cohort-level methylation quality control (QC); (5) participants passed cohort-specific standard QCs (for example, genetic outliers were excluded); and (6) participants were not disease cases from a case/control study.

DNA methylation measurement and cohort-level QC

Whole-blood DNA CpG methylation was measured genome-wide in all cohorts using the Illumina 450k Human Methylation chip (San Diego, CA, USA). We standardized the cohort-level QC and preprocessing of the methylation data as much as possible, while ensuring some degree of flexibility to keep the implementation feasible for all cohorts (leading to slight variation in preprocessing across cohorts, as is common^{13,15,17}). Cohort-specific information regarding technical treatment of the data, such as background-correction,³⁶ normalization³⁷ and QC, is reported in Supplementary Table S1.4.

Statistical analysis

Our analyses were performed in accordance with a preregistered analysis plan archived at Open Science Framework (OSF) in September 2015 (available at: <https://osf.io/9v3nk/>).

Epigenome-wide association study

To investigate associations with individual methylation markers we first performed cohort-level EWAS of EA that we subsequently meta-analysed (Supplementary Note 2). As is standard, the EWAS was performed as a set of linear regressions in each cohort, one methylation marker at a time, with the methylation beta value (0–1) as the dependent variable. The key independent variable was EA. We estimated two regression models that differ in the set of covariates included. In the basic model, the covariates were age, sex, imputed or measured white blood cell counts, technical covariates from the methylation array and four genetic principal components to account for population stratification. In the adjusted model, we additionally controlled for BMI (kg/m²), smoker status (three categories: current, previous or never smoker), an interaction term between age and sex and a quadratic term for age. As BMI and smoking are correlated with EA^{38,39} and known to be associated with methylation,^{13,17} the basic model may identify associations with EA that are actually due to BMI or smoking. Although the adjusted model reduces that risk, it may also reduce power to identify true associations with EA (by controlling for factors that are correlated with EA). Although we present the results for both models, we focus on the adjusted model because it is more conservative. Details of cohort-specific covariates are presented in Supplementary Table S1.4.

EWAS QC and meta-analysis

Each participating cohort uploaded EWAS summary statistics to a central secure server for QC and meta-analysis. The number of CpG probes filtered at each step of the QC is presented in Supplementary Table S1.5. We removed: probes with missing *P*-value, s.e. or coefficient estimate; probes with a call rate < 95%; probes with a combined sample size < 1000; probes not available in the probe-annotation reference by Price *et al.*;⁴⁰ CpH probes (H = A/C/T); probes on the sex chromosomes; and cross-reactive probes highlighted in a recent paper by Chen *et al.*⁴¹ We performed a sample-size-weighted meta-analysis of the cleaned results using METAL.⁴² We used single genomic control, as is common in genome-wide association studies (GWAS),⁴³ to stringently correct the meta-analysis *P*-values for possible unaccounted for population stratification.⁴⁴ Probes with a *P*-value < 1×10^{-7} , a commonly used threshold in EWAS¹ that we prespecified in the analysis plan, were considered epigenome-wide significant associations.

Epigenetic clock analyses

To construct our epigenetic clock variables (Supplementary Note 3), the cohort-level raw beta-value data were entered into the online Horvath calculator,²³ as per our preregistered analysis plan. The 'normalize data' and 'advanced analysis for Blood Data' options were selected. The following variables were selected from the calculator's output for subsequent analysis:

- Clock 1. Horvath age acceleration residuals, which are the residuals from the regression of chronological age on Horvath age.
- Clock 2. White blood cell count-adjusted Horvath age acceleration, which is the residual from Clock 1 after additional covariate adjustment for imputed white blood cell counts.
- Clock 3. White blood cell count-adjusted Hannum age acceleration, which is the same as Clock 2 but with the Hannum age prediction in place of the Horvath prediction.
- Clock 4. Cell count-enriched Hannum age acceleration, which is the basic Hannum predictor plus a weighted average of aging-associated cell counts. This index has been found to have the strongest association with mortality.⁴⁵

These Clock measures are annotated in the Horvath software as follows: 'AgeAccelerationResidual', 'AAHOAdjCellCounts', 'AAHAAdjCellCounts', and 'BioAge4HAAdjAge'. We analyzed two

regression models, both with EA as the dependent variable and a clock variable as an independent variable. In the basic age acceleration model, we control for sex, and in the adjusted age acceleration model, we additionally control for BMI and smoker status (current, previous or never smoker). In total, in each adult cohort, we estimated eight regressions: each of the two models with each of the four clock variables as an independent variable. For each of the eight regressions, we performed a sample-size-weighted meta-analysis of the cohort-level results.

Polygenic predictions with polygenic methylation score

We performed a prediction analysis with polygenic methylation scores (PGMSs), analogous to polygenic score prediction in the GWAS literature (Supplementary Note 6). We tested the predictive power in three independent adult cohort studies: Lothian Birth Cohort 1936 (LBC1936, *n* = 918), RS-BIOS (Rotterdam Study—BIOS, *n* = 671), and RS3 (Rotterdam Study 3, *n* = 728). We re-ran the EWAS meta-analysis for each prediction cohort to obtain the weights for the PGMS, while holding out the prediction cohort to avoid overfitting. We constructed the PGMS for each individual as a weighed sum of the individual's methylation markers' beta values and the EWAS effect-sizes, using the *Z*-statistics from the EWAS as weights. (The *Z*-statistics were used instead of the EWAS coefficients because CpG methylation is the dependent variable in the EWAS regression.) We constructed PGMSs using two different thresholds for probe inclusion, $P < 1 \times 10^{-5}$ and $P < 1 \times 10^{-7}$, with weights from the basic and adjusted EWAS models, for a total of four PGMSs in each prediction cohort.

To shed light on the direction of causation of epigenetic associations, we used a fourth prediction cohort study, a sample of children in the ALSPAC ARIES cohort.⁴⁷ We constructed the PGMS using the same approach as described above, in this case using data from cord-blood-based DNA methylation at birth. The outcome variables in this cohort were average educational achievement test scores (Key Stages 1–4⁴⁸) from age 7 up to age 16 years.

To examine the relationship between epigenetic and genetic associations, we also constructed a single-nucleotide polymorphism polygenic score (SNP PGS) for EA. We used SNP genotype data available in the three adult prediction cohort studies (LBC1936, RS-BIOS and RS3). We constructed the SNP PGS in each cohort as a weighted sum of the individual genotypes from all available genotyped SNPs, with GWAS meta-analysis coefficients as weights. We obtained the coefficients by re-running the largest GWAS meta-analysis to date of EA³⁵ after excluding our prediction cohorts (LBC1936, RS-BIOS and RS3).

We evaluated the predictive power of the PGMS by examining the incremental coefficient of determination (incremental R^2) for predicting EA (or test scores in ALSPAC ARIES). The incremental R^2 is the difference in R^2 between the regression model with only covariates and the same regression model that additionally includes the PGMS as a predictor. The covariate-only models in the LBC1936, RS-BIOS and RS3 cohorts controlled for age, sex and the SNP PGS. In the ALSPAC ARIES cohort, we controlled for age at assessment and sex. In the ALSPAC ARIES cohort, when we investigate maternal smoking as a potential confound for our EA associations, we add maternal smoking to the set of covariates. We finally restricted the ALSPAC ARIES cohort to children with non-smoking mothers. To investigate a possible interaction effect between the PGMS and SNP PGS, we re-estimated the regression model after adding an interaction term between the PGMS and the SNP PGS, and the incremental R^2 was calculated as the difference in R^2 relative to the model that included the PGMS and the SNP PGS as additive main effects.

RESULTS

Descriptive statistics

Summary statistics from the 27 independent cohort studies from the 15 contributing cohorts are shown in Supplementary Table

S1.1. The mean age at reporting ranges from 26.6 to 79.1 years, and the sample size ranges from 48 to 1658, with a mean of 399 individuals per cohort. The mean cohort EA ranges from 8.6 to 18.3 years of education, and the sample-size-weighted mean is 13.6 (s.d.=3.62). The meta-analysis sample is 54.1% female.

Epigenome-wide association study

EWAS quality control. The QC filtering is reported in Supplementary Table S1.5. We inspected the quantile–quantile (QQ) plot of the filtered EWAS results from each contributing cohort as part of the QC procedure before meta-analysis. The genomic inflation factor (λ_{GC}), defined as the ratio of the median of the empirically observed chi-square test statistics to the expected median under the null, had a mean across the cohorts of 1.02 for the adjusted model (s.d.=0.18). We report the cohort-level genomic inflation factor after probe filtering in Supplementary Table S1.5. The variation in λ_{GC} across cohorts was comparable to that from EWAS performed in cohorts of similar sample size.¹² We applied genomic control at the cohort level, which is a conservative method of controlling for residual population stratification that may remain even despite the regression controls for principal components.⁴⁴ The meta-analysis λ_{GC} was 1.19 for the basic model and 1.06 for the adjusted model.

EWAS results. Figure 1 shows the Manhattan plot for the meta-analysis results of the adjusted model. The Manhattan plot for the basic model is reported in Supplementary Note, together with the QQ plots for the basic and adjusted model. In the basic model, there were 37 CpG probes associated with EA at our preregistered epigenome-wide P -value threshold ($P < 1 \times 10^{-7}$); these results are reported in Supplementary Table S1.6a. In the adjusted model, there were nine associated probes, listed in Table 1 (with additional details in Supplementary Table S1.7a), all of which were also associated in the basic model. We hereafter refer to the adjusted model's nine associated probes as the 'lead probes'. In Supplementary Note 2.4.2, we present the association results with false discovery rate < 0.05 , but as this threshold was not prespecified in the analysis plan, we do not present these results as main findings.

To investigate how the EWAS results look at a regional level, we analyzed the distribution of the EWAS associations across the genome by performing enrichment tests for methylation density regions⁴⁰ (the so-called 'HIL' categories; Supplementary Note 5.2).

We found that the number of probes with $P < 1 \times 10^{-7}$ is more or less proportional to the total number of probes in every region and that there is enrichment for association in all four methylation density categories: high-density CpG islands (HC), intermediate-density CpG islands (IC), intermediate-density CpG islands bordering HCs (ICshore), and non-islands (LC).

The effect sizes of the associations for the nine lead probes are shown in Table 1. The coefficients of determination (R^2 s) range from 0.3% to 0.7%. To put these effect sizes in perspective, Figure 2 and Supplementary Table S1.8 compare the R^2 s for the top 50 probes in our adjusted model with the top 50 probes from recent large-scale EWAS of smoking,¹³ maternal smoking,¹² alcohol consumption¹⁵ and BMI,¹⁷ as well as the top 50 GWAS SNP associations with EA.³⁵ The EA EWAS associations of our study are an order of magnitude larger than the largest EA SNP effect sizes. However, our EWAS associations are small in magnitude relative to the EWAS associations reported for more biologically proximal environmental factors. BMI is the most similar to EA, with R^2 s of associated probes approximately 20–50% larger than those for EA. Relative to the largest R^2 for an EA-associated probe, the largest effect for probes associated with smoking and maternal smoking are greater by factors of roughly 3 and 17, respectively.

Lookup of lead probes in the published EWAS of smoking. As our smoker-status control variable is coarse and discrete (current, former or never smoker), we were concerned that the adjusted EWAS model might not have adequately controlled for exposure to smoking (that is, amount and duration of smoking and exposure to second-hand smoke). Therefore, we performed a lookup of our lead probes in the published EWAS on smoking (Supplementary Note 4 and Supplementary Table S1.10). We found that all nine lead probes have previously been associated with smoking. The results of this lookup motivated our analysis of the never-smoker subsample, discussed next.

Robustness of EWAS results in the never-smoker subsample. To minimize the possible confounding effect of smoking on the association between EA and CpG methylation, we conducted a set of analyses that we did not anticipate when we preregistered our analysis plan. Specifically, we went back to the cohorts and asked them to re-conduct their EWAS, this time restricting the analysis to individuals who self-reported to be never smokers. After following the same QC steps as above, we performed a new meta-analysis of these results ($n = 5175$).

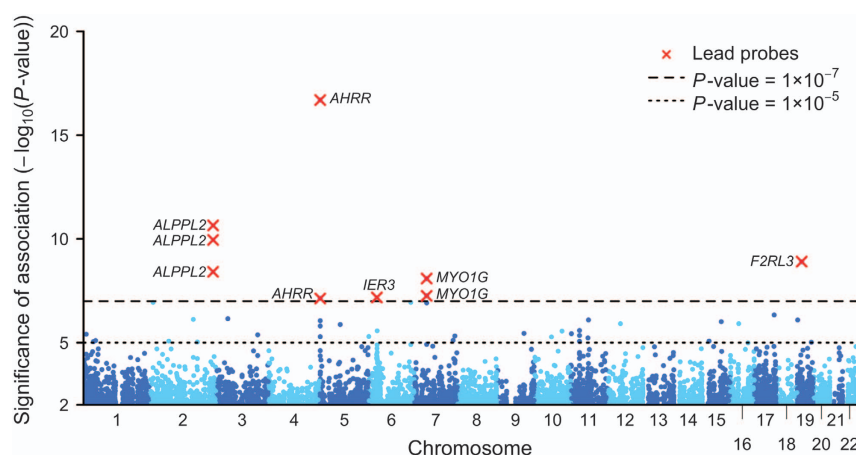


Figure 1. Manhattan plot of the adjusted epigenome-wide association study (EWAS) model. The figure displays the Manhattan plot of the meta-analysis of the adjusted EWAS model (the Manhattan plot of the basic model is reported in Supplementary Note). The x axis is the chromosomal position, and the y axis is the significance on a $-\log_{10}$ scale. The dashed lines mark the threshold for epigenome-wide significance ($P = 1 \times 10^{-7}$) and for suggestive significance ($P = 1 \times 10^{-5}$). Each epigenome-wide associated probe is marked with a red x and the symbol of the closest gene based on physical position.

Table 1. EWAS association results—adjusted model

EWAS association results—adjusted model (9 probes with P-value $< 1 \times 10^{-7}$)										
Probe	Chr:Position (GRCh37)	n	P-value	R ²	Closest gene	Distance closest gene TSS	Expected power in never-smokers (n=5175)	n (never-smokers)	P-value (never-smokers)	R ² (never-smokers)
cg05575921	5:373379	10 315	2.03×10^{-17}	0.70%	AHRR	−47 656	75.1%	5174	1.46×10^{-1}	0.04%
cg21566642	2:233284662	9633	2.26×10^{-11}	0.46%	ALPPL2	13 110	33.3%	4627	6.36×10^{-2}	0.07%
cg05951221	2:233284403	10 313	1.12×10^{-10}	0.40%	ALPPL2	12 851	22.2%	5174	3.10×10^{-3}	0.17%
cg03636183	19:17000586	10 313	1.24×10^{-9}	0.36%	F2RL3	760	15.2%	5172	6.55×10^{-1}	0.00%
cg01940273	2:233284935	10 316	3.84×10^{-9}	0.34%	ALPPL2	13 383	12.3%	5175	3.37×10^{-2}	0.09%
cg12803068	7:45002920	10 316	8.09×10^{-9}	0.32%	MYO1G	6067	10.6%	5174	1.48×10^{-4}	0.28%
cg22132788	7:45002487	9531	5.52×10^{-8}	0.31%	MYO1G	6500	9.2%	4334	4.35×10^{-4}	0.28%
cg06126421	6:30720081	9718	6.63×10^{-8}	0.30%	IER3	−7753	8.2%	5174	2.98×10^{-1}	0.02%
cg21161138	5:399361	10 309	7.39×10^{-8}	0.28%	AHRR	−21 674	6.4%	5170	6.59×10^{-2}	0.07%

Abbreviations: EWAS, epigenome-wide association study; TSS, transcription start site. Note: 'Distance closest gene TSS' measured in base pairs. An extended version of this table is available in Supplementary Table S1.7a.

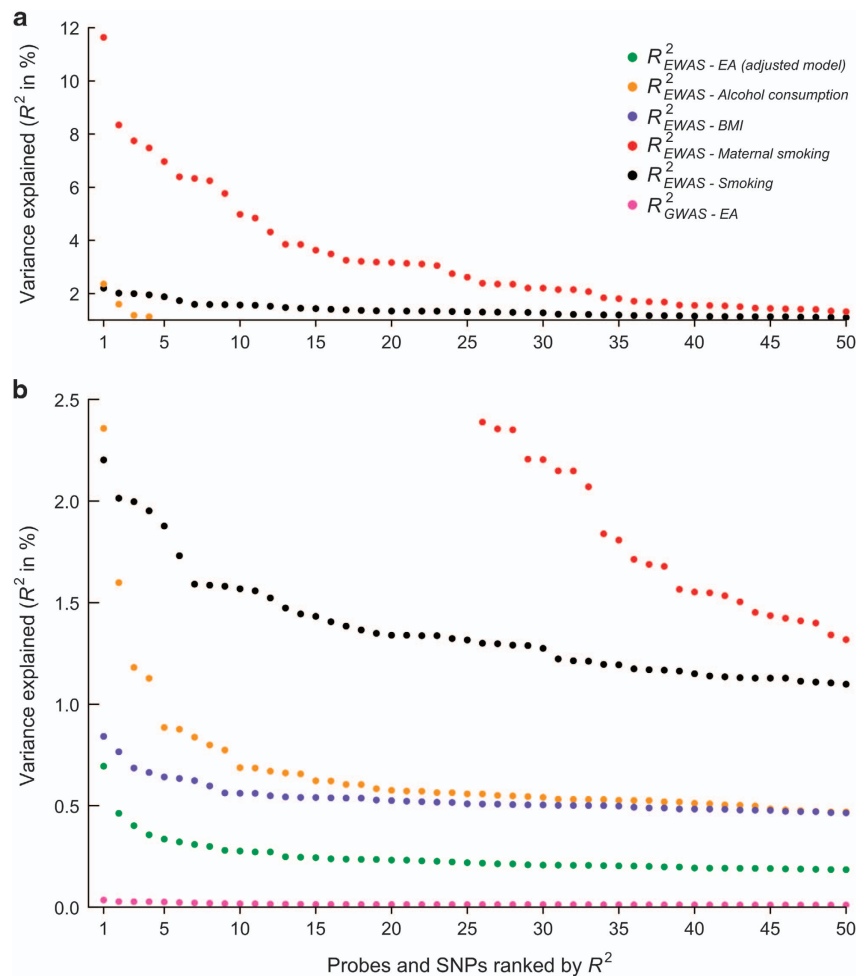


Figure 2. Epigenome-wide association study (EWAS) effect sizes (in terms of variance explained) across traits and with genome-wide association study (GWAS). The figure displays the effect size estimates in terms of R^2 , in descending order, for the 50 top probes of the adjusted EWAS model. For comparison, we present the 50 top probes from recent EWAS on alcohol consumption ($n=9643$, Liu *et al.*, 2016), body mass index (BMI; $n=7798$, Mendelson *et al.*, 2017), smoking ($n=9389$, Joehanes *et al.*, 2016) and maternal smoking ($n=6685$, Joubert *et al.*¹²). For comparison with GWAS effect sizes, we contrast the EWAS probes with the effect sizes of the 50 top approximately independent single-nucleotide polymorphisms (SNPs) from a recent GWAS on educational attainment (EA; $n=405\,073$, Okbay *et al.*, 2016). Panels (a and b) display the same results but with a different scaling of the y axis in order for the smaller effect sizes to be visible.

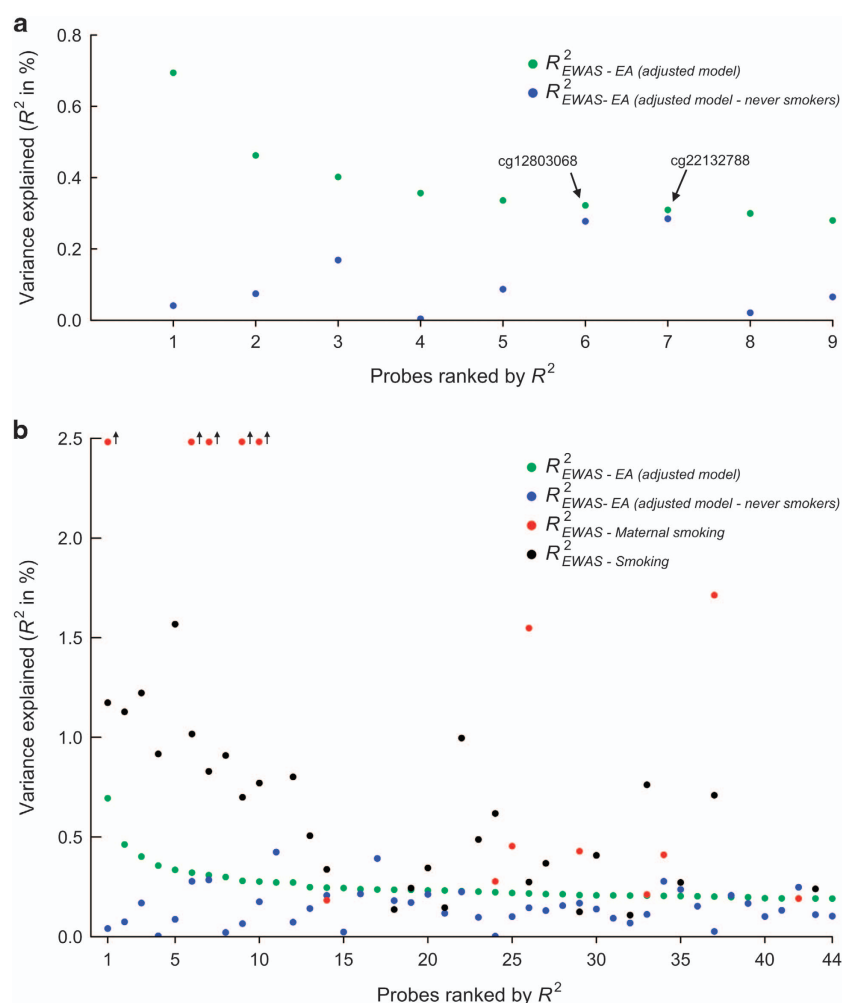


Figure 3. Comparison of educational attainment (EA) epigenome-wide association study (EWAS) effect sizes with the effect sizes in the never-smoker subsample and in smoking EWAS results. Panel (a) displays the effect-size estimates in terms of R^2 for the nine lead probes, in descending order, and the lead probe's corresponding effect size when re-estimated in the subsample of never smokers. Panel (b) displays the same information for the probes of the adjusted model with $P < 1 \times 10^{-5}$ (including the nine lead probes), as well as the same probes' effect-size estimates from two recent EWAS of smoking ($n = 9389$, Joehanes *et al.*¹³), and maternal smoking ($n = 6685$, Joubert *et al.*¹²). The smoking and maternal smoking estimates are only publicly available for probes associated at false discovery rate < 0.05 in the respective EWAS.

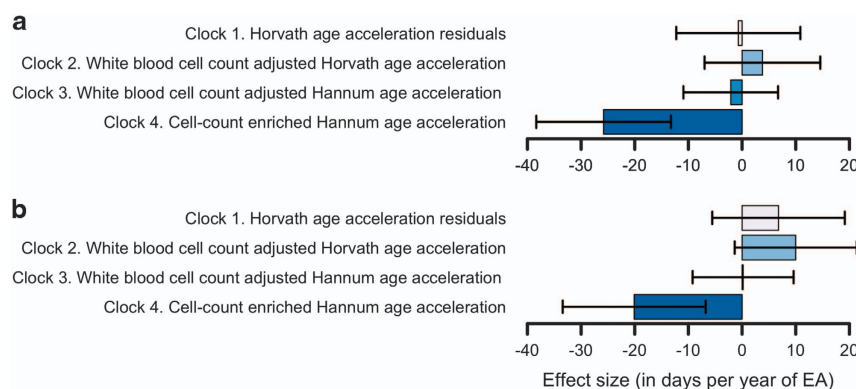


Figure 4. Effect-size estimates (in days) of the epigenetic clock analyses with 95% confidence intervals. Panel (a) displays the effect-size estimates from the basic age acceleration model, and panel (b) displays the effect-size estimates from the adjusted age acceleration model. The effect size is denoted in days of age acceleration per year of educational attainment, and error bars represent 95% confidence intervals.

In this subsample, the effect-size estimates were smaller by at least 60% for 7 of the 9 lead probes (see Table 1 and Figure 3a), whereas two probes (cg12803068 and cg22132788) had similar effect-size estimates as in the full sample (statistically

distinguishable from 0 with $P = 1.48 \times 10^{-4}$ and $P = 4.35 \times 10^{-4}$, respectively). These two probes, however—both in proximity to the gene *MYO1G*—have been found to be associated with maternal smoking during pregnancy, and the effects on the

methylation of this gene are persistent when measured at age 17 years in the offspring.^{12,49} This influence has been shown to continue through to middle age.⁵⁰ We cannot distinguish between the hypothesis that these probes have some true association with EA and the hypothesis that their apparent association with EA is entirely driven by more maternal smoking during pregnancy among lower EA individuals. We also cannot rule out that the probes' association with EA is driven by second-hand smoke exposure, which could also be correlated with EA.

To assess how widely such confounding may affect the EA results, in Figure 3b we compare the effect sizes of all the probes associated with EA at $P < 1 \times 10^{-5}$ in the adjusted EWAS model to the effect sizes found for the same probes in EWAS meta-analyses of smoking¹³ and maternal smoking¹² (see also Supplementary Table S1.11). Many of the EA-associated probes are also associated with smoking or maternal smoking, strongly suggesting that residual smoking exposure (that is, the misclassification of amount and duration of smoking and second-hand smoke that is not captured by the smoking covariate) and maternal smoking remain potential confounding factors for the probe associations with EA, even in the subsample of individuals who are self-reported never smokers.

Epigenetic clock associations with EA. Two cohorts, FINRISK and MCCS, did not contribute to the epigenetic clock analyses. Therefore, the sample sizes for these analyses were smaller than for the EWAS meta-analysis: 8173 for the basic age acceleration model and 7691 for the adjusted age acceleration model (the difference being due to missing covariates for some individuals). The effect-size estimates are presented in Figure 4 and Supplementary Table S1.9. There was no evidence for an association between EA and Clocks 1, 2 or 3, but the association between EA and Clock 4 was strong ($P = 3.51 \times 10^{-6}$ and $P = 4.51 \times 10^{-4}$ in the basic and adjusted age acceleration models, respectively). The point estimates were small, however: using Clock 4, each year of EA was associated with a 0.071-year (that is, 26-day) reduction in age acceleration in the basic model and a 0.055-year (that is, 20-day) reduction in the adjusted model. Overall then, higher EA was associated with slightly younger biological age when compared with chronological age. We note that the epigenetic clock that was found to be associated with EA, Clock 4, has previously been found to be the most predictive epigenetic clock measure of mortality,⁴⁵ and an independent study also found a negative association between Clock 4 and EA⁴⁶

Prediction using PGMSs. The incremental R^2 s from the prediction of EA with PGMSs in our adult prediction cohort studies, the LBC1936, RS-BIOS and RS3, are reported in Supplementary Table S1.13a and Figure 5. Across the four PGMSs constructed with weights from the basic and adjusted model, and with the two probe-inclusion thresholds ($P < 1 \times 10^{-5}$ and $P < 1 \times 10^{-7}$), the incremental R^2 s ranged from 1.4% to 2.0% ($P \leq 3.28 \times 10^{-8}$). There was also weak evidence for an interaction between the PGMS and the SNP PGS in predicting EA, with the R^2 s for the interaction term ranging from 0.1% to 0.3% (P -values ranged from 0.01 to 0.12).

In the subsample of never smokers, the PGMSs (constructed with weights derived from the full EWAS sample), the PGMS is far less predictive, with incremental R^2 s ranging from 0.3% to 0.9% (Figure 5 and Supplementary Table S1.13b). The two PGMSs constructed from probes with $P < 1 \times 10^{-5}$ in the EWAS were associated with EA at $P < 0.05$, while the two PGMSs constructed only from the lead probes with $P < 1 \times 10^{-7}$ were not ($P > 0.05$). No interaction effect was found between the PGMS and the SNP PGS in the never-smoker subsample.

To further investigate confounding by smoking in the prediction analysis, we examined the correlations between our PGMSs constructed from the lead probes (that is, those associated with EA at significance threshold $P < 1 \times 10^{-7}$) in either our basic or

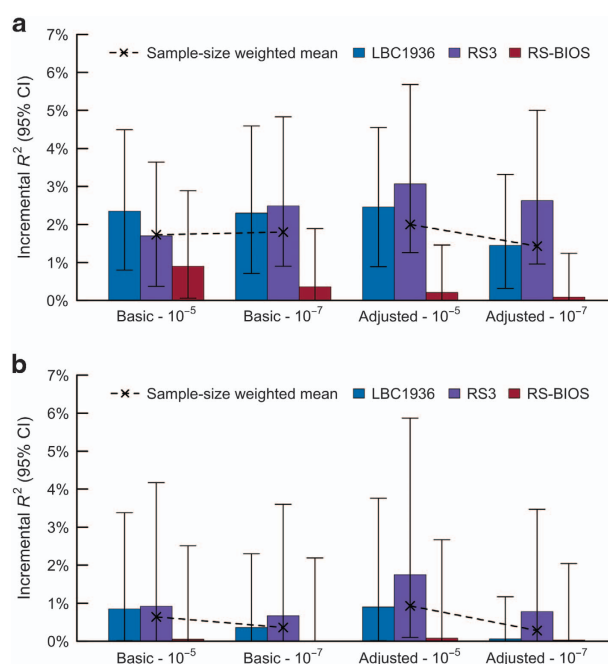


Figure 5. Methylation score prediction of educational attainment in independent holdout samples. Panel (a) displays the prediction in all individuals, and panel (b) displays the prediction in the subsample of never smokers. Four methylation scores were constructed using coefficient estimates from the basic model versus adjusted model, crossed with a P -value threshold of 1×10^{-5} and 1×10^{-7} . The sample sizes of the LBC1936, the RS3 and the RS-BIOS cohorts are 918, 728 and 671 individuals, respectively. We performed sample-size-weighted meta-analysis across the cohorts for each of the four methylation score prediction analyses. From left to right, the respective P -values testing the null hypothesis of zero predictive power are 4.42×10^{-11} , 7.76×10^{-11} , 2.02×10^{-11} and 3.28×10^{-8} for the full sample and 0.0183, 0.0898, 0.0051 and 0.1818 for the never smokers, respectively. The full prediction results are presented in Supplementary Tables S1.9a and b.

adjusted model and a PGMS for smoking (see Supplementary Note 6.2.2 for details). For the smoking PGMS, we use the 187 probes that were identified at epigenome-wide significance ($P < 1 \times 10^{-7}$) and then successfully replicated in a recent EWAS of smoking.⁵¹ We examine the PGMS correlations in our full prediction samples, not restricted to never smokers. For the EA PGMS from our basic model, we find a correlation with the smoking PGMS of -0.96 in RS3, -0.94 in RS-BIOS and -0.93 in LBC1936. For the EA PGMS from our adjusted model, the correlations are -0.90 , -0.89 and -0.91 , respectively. In all cases, the nearly perfect correlation between the smoking and EA methylation scores strongly suggests that smoking status confounds the EWAS associations with EA.

Turning to the child sample in the ALSPAC ARIES cohort,^{47,49} we examined whether a PGMS constructed from methylation assessed in cord blood samples at birth was predictive of four prospective measures of educational achievement test scores (Key Stage 1–4⁴⁸), collected between ages 7 and 16 years (Supplementary Note 6.1.1). The results are reported in Supplementary Table S1.13c. The largest incremental R^2 was 0.73% ($P = 0.0094$), and it was attained in the model predicting school performance at age 14–16 years (that is, the Key Stage 4 test scores). However, once maternal smoking status was added as a control variable, the predictive power of the PGMS became essentially zero (incremental $R^2 = 0.05\%$, $P = 0.234$). This suggests that the confounding effects of maternal smoking strongly influenced the predictive power of the PGMS for EA. We draw

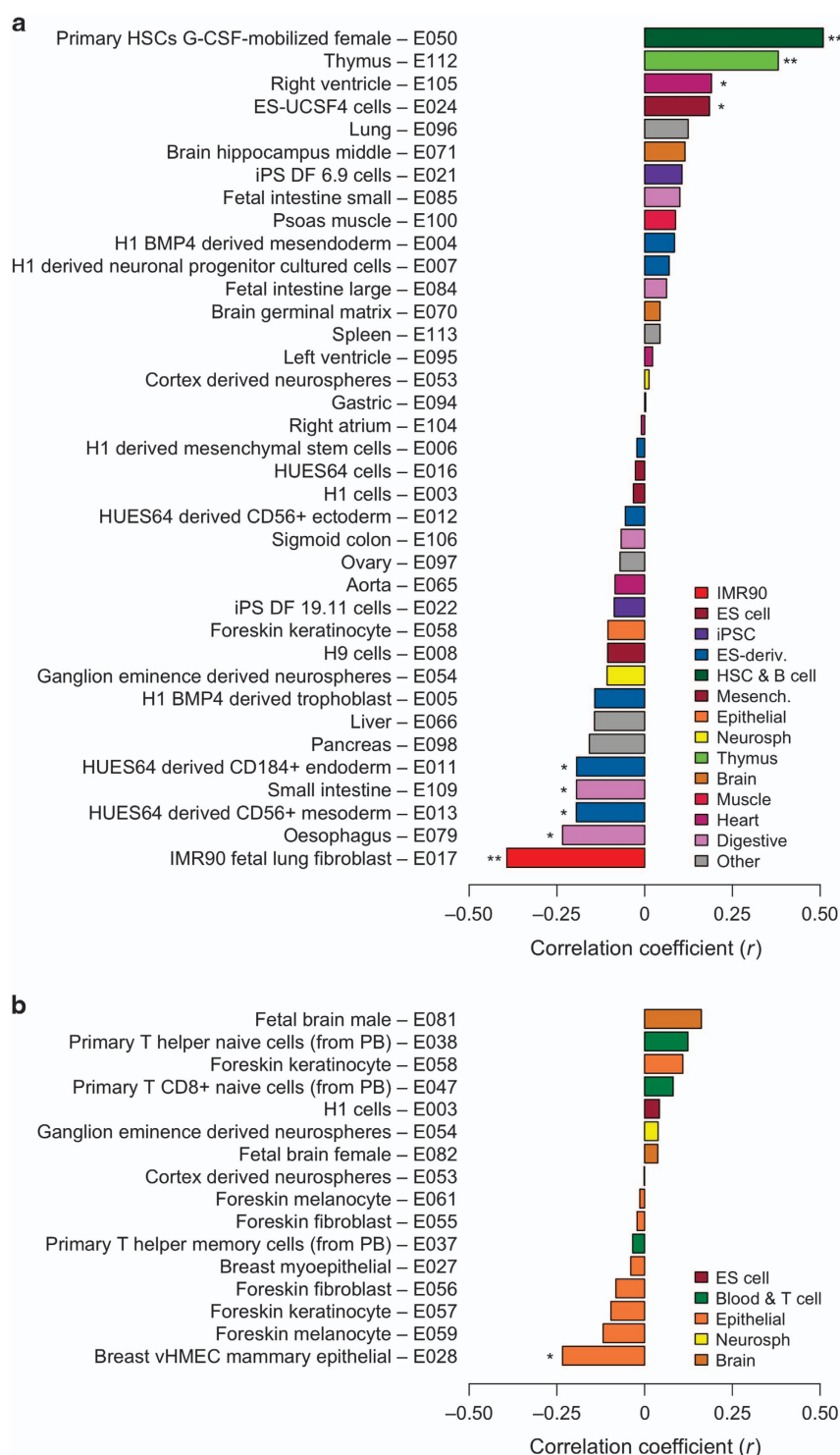


Figure 6. Correlations between tissue-specific methylation and the epigenome-wide association study association results (adjusted model). Panel (a) displays the correlation estimates based on the whole-genome bisulfite sequencing methylation measurement, and panel (b) displays results based on the mCRF methylation measurement. (The mCRF measurement combines sequencing data from the MeDIP-seq and MRE-seq methods.) The method is described in Supplementary Note 7. Correlations that are significant after Bonferroni correction are marked with two asterisks (**), and marginal significance ($P < 0.05$) is marked with one asterisk (*). The tissue-specific methylation data are from the Roadmap Epigenomics Consortium, and we used their categorization and color code for simplicity of comparison.⁵²

two conclusions from these results from the child sample. First, they reinforce the concern that maternal smoking was a major confound for any probe associations with EA. Second, they suggest that any true methylation–EA associations were unlikely to be driven by a causal effect of methylation on EA.

Overlap between EWAS probes and published GWAS associations. To supplement our polygenic score analyses of the overlap between epigenetic and genetic associations, we next investigated whether our lead probes are located at loci that contain SNPs previously identified in GWAS of EA and smoking

(Supplementary Note 5). Considering jointly the 141 approximately independent EWAS probes with $P < 1 \times 10^{-4}$, we did not find evidence of enrichment for either EA-linked SNPs ($P = 0.206$) or smoking-linked SNPs ($P = 0.504$). Considering the probes individually, one probe (cg17939805) was found to be in the same genomic region as a SNP (rs9956387) associated with EA (with a genomic distance of 607 bp), whereas no probes were close to SNPs previously identified as linked to smoking.

Correlation of EWAS results with tissue-specific methylation. To answer the question of whether our EWAS associations are correlated with any tissue-specific DNA methylation, we utilized the tissue-specific methylation data made available by the Epigenomic Roadmap Consortium.⁵² Their data were used to calculate tissue-specific deviations from average cross-tissue methylation at the loci corresponding to the EWAS CpG probes associated with EA at a P -value $< 1 \times 10^{-4}$ (Supplementary Note 7). We examined the correlation between these tissue-specific deviations and the EWAS association test statistics (Z -statistics) of the probes, using the results from the adjusted EWAS model. We report the results in Figure 6 and in Supplementary Table S1.14. The strongest correlations were found for primary haematopoietic stem cells granulocyte colony-stimulating factor-mobilized female and IMR90 fetal lung fibroblast. Intermediate strength correlations were found across multiple, seemingly unrelated tissues, while no correlations of relevant magnitudes were found with the brain tissues available in the Roadmap. We interpret the lack of correlation with tissues plausibly related to EA (such as brain tissues) as supporting the conclusion that the EWAS results are driven by confounding factors rather than by a true association with EA.

Pathway analysis with gene-expression data. Using the GTEx⁵³ expression data and the webtool 'functional mapping and annotation of genetic associations' (FUMA)⁵⁴ we performed a pathway analysis. The analysis used the GTEx gene-expression levels to cluster the 29 genes physically closest to the EA-associated (at $P < 1 \times 10^{-5}$) CpG probes of the adjusted model (Supplementary Note 8). The results of the expression analysis are displayed in Supplementary Figure 4. We find that the genes closest to the EA-associated probes are expressed across multiple tissues that have no clear relationship to EA (such as blood tissues, among many other); for further discussion, see Supplementary Note. Overall, these results are consistent with the hypothesis that the EWAS results are driven by confounding factors.

DISCUSSION

We believe this study provides one of the first large-scale investigations in humans of epigenetic changes linked to a biologically distal environmental factor. In our EWAS meta-analysis—one of the largest EWAS conducted to date—we found nine CpG probes associated with EA. Each of these probes explains 0.3–0.7% of the variance in EA—effect sizes somewhat smaller than the largest EWAS effects that have been observed for BMI and many times smaller than those observed for alcohol consumption, smoking and especially maternal smoking during pregnancy. When we restrict our analysis to the subsample of never smokers, the effect sizes of seven out of the nine lead probes are substantially attenuated. Moreover, the other two lead probes have been found in previous work to be strongly associated with maternal smoking during pregnancy.¹² More generally, comparing our own results to those from previous EWAS highlights a variety of factors correlated with EA, including not only maternal smoking but also alcohol consumption and BMI, as potentially major confounding factors for the EA associations we detect. We also cannot rule out that other factors correlated

with EA, such as exposure to second-hand smoke, could confound the EA associations. This should be taken into account in future endeavours of associating methylation with biologically distal factors that are known to correlate with environmental factors that have a fairly direct biological impact, such as smoking.

Convincingly establishing a causal effect of EA would require analyzing a sample with quasi-random variation in EA, such as a sample in which some individuals were educated after an increase in the number of years of compulsory schooling and other individuals were educated before the law change.⁵⁵ We are not aware of large EWAS samples with quasi-random variation at present, but we anticipate that such samples will become available as methylation becomes more widely measured.

Although the EWAS we report here is among the largest conducted to date, our sample size of 10 767 individuals is only large enough to identify nine probes associated with EA at the conventional epigenome-wide significance threshold. Subsequent EWAS conducted in larger samples that have sufficient statistical power to identify a much larger number of EA-associated probes will enable more extensive investigations of overlap with probes associated with other phenotypes than were possible from our results, as well as analyses of the biological functions of the probes. Besides limited statistical power, other limitations of our study, common to EWAS research designs, are that we study methylation cross-sectionally and not longitudinally and that we only investigate CpG methylation and not other types of epigenetic modifications. Also, our study focuses on single CpG sites; future studies could consider additional analytical approaches to assess regions of differential methylation (for example, genes). Once suitable methods have been developed, it would also be of interest to estimate the overall proportion of variance in EA that can be attributed to individual differences in DNA methylation patterns.

CONCLUSION

One plausible hypothesis is that environmental influences on the epigenome—even those due to everyday, social environmental factors—are pervasive and profound.³ According to the logic of this view, a major life experience that occurs over many years, such as EA, should leave a powerful imprint on the epigenome. Motivated by this view and by the evidence of large EWAS effects in studies of lifestyle factors, when we embarked on this project we entertained the hypothesis that we might find large associations between EA and methylation. We also entertained the alternative hypothesis that EA, because it is so biologically distal, may exhibit much weaker associations with methylation.

Although our results do not allow us to distinguish how much of the effects we find are due to true associations with EA and how much are due to confounding factors, they strongly suggest that the effect sizes we estimate are an upper bound on the effect sizes of any true methylation associations with EA. These upper-bound effect sizes are far smaller than associations with more biologically proximal environmental factors that have been studied. If our results can be generalized beyond EA to other biologically distal environmental factors, then they cast doubt on the hypothesis that such factors have large effects on the epigenome.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was carried out under the auspices of the Social Science Genetic Association Consortium (SSGAC). The SSGAC seeks to facilitate studies that

investigate the influence of genes on human behavior, well-being and social-scientific outcomes using large genome-wide association study meta-analyses. The SSGAC also provides opportunities for replication and promotes the collection of accurately measured, harmonized phenotypes across cohorts. The SSGAC operates as a working group within the CHARGE consortium. A full list of acknowledgments is provided in Supplementary Note. Upon publication, results can be downloaded from the SSGAC website (<http://www.thessgac.org/>). Data for our analyses come from many studies and organizations, some of which are subject to a MTA, and are listed in the Supplementary Note. We thank Aysu Okbay for conducting the meta-analyses for the SNP polygenic scores. The data were accessed under Section 4 of the Data Sharing Agreement of the SSGAC.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)